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(54) Title: METHODS AND MEANS FOR INDUCING APOPTOSIS BY INTERFERING WITH BIP-LIKE PROTEINS

(57) Abstract

The invention relates to activation of apoptosis by means of interference of the function of Bip-like compounds. Also the invention relates to anti-tumor therapies with compounds, which negatively interfere with Bip-like compounds leading to induction of apoptosis, resulting in the elimination of tumor cells. Also the invention relates to therapies for diseases related to aberrant apoptosis induction, such as auto-immune diseases. Also the invention describes the diagnosis of cells, which are susceptible to apoptin or apoptin-like induces apoptosis.

Title: Methods and means for inducing apoptosis by interfering with Bip-like proteins.

The present invention relates to the field of apoptosis, as well as to the field of cancer diagnosis and treatment, and treatment and diagnosis of auto-immune diseases and other diseases by induction of apoptosis. In particular the invention provides novel molecules and means to induce apoptosis or enhance apoptosis. The novel molecules and means are part of the apoptotic pathway induced by apoptin. Apoptin is a protein originally found in chicken anemia virus (CAV; Noteborn et al., 1991) and was originally called VP3. The apoptotic activity of this protein was discovered by the group of the present inventors (Noteborn et al., 1994).

As stated above the present invention makes use of the induction of apoptosis in which Bip-like proteins are involved.

Apoptosis is an active and programmed physiological process for eliminating superfluous, altered or malignant cells (Earnshaw, 1995, Duke et al., 1996). Apoptosis is characterized by shrinkage of cells, segmentation of the nucleus, condensation and cleavage of DNA into domain-sized fragments, in most cells followed by internucleosomal degradation. The apoptotic cells fragment into membrane-enclosed apoptotic bodies. Finally, neighbouring cells and/or macrophages will rapidly phagocytose these dying cells (Wyllie et al., 1980, White, 1996). Cells grown under tissue-culture conditions and cells from tissue material can be analysed for being apoptotic with agents staining DNA, as e.g. DAPI, which stains normal DNA strongly and regularly, whereas apoptotic DNA is stained weakly and/or irregularly (Noteborn et al., 1994, Telford et al., 1992).

The apoptotic process can be initiated by a variety of regulatory stimuli (Wyllie, 1995, White 1996, Levine, 1997). Changes in the cell survival rate play an important role in human pathogenesis, e.g. in cancer development, which is

caused by enhanced proliferation but also by decreased cell death (Kerr et al., 1994, Paulovich, 1997). A variety of chemotherapeutic compounds and radiation have been demonstrated to induce apoptosis in tumor cells, in many instances via wild-type p53 protein (Thompson, 1995, Bellamy et al., 1995, Steller, 1995, McDonell et al., 1995).

Many tumors, however, acquire a mutation in p53 during their development, often correlating with poor response to cancer therapy. Transforming genes of tumorigenic DNA viruses inactivate p53 by directly binding to it (Teodoro, 1997). An example of such an agent is the large T antigen of the tumor DNA virus SV40. For several (leukemic) tumors, a high expression level of the proto-oncogene Bcl-2 or Bcr-abl is associated with a strong resistance to various apoptosis-inducing chemotherapeutic agents (Hockenberry 1994, Sachs and Lotem, 1997).

For such cancers (representing more than half of the tumors) alternative anti-tumor therapies are under development based on induction of apoptosis independent of p53 (Thompson 1995, Paulovich et al., 1997). One has to search for the factors involved in induction of apoptosis, which do not need p53 and/or can not be blocked by Bcl-2/Bcr-abl-like anti-apoptotic activities. These factors might be part of a distinct apoptosis pathway or being (far) downstream to the apoptosis inhibiting compounds.

Apoptin is a small protein derived from chicken anemia virus (CAV; Noteborn and De Boer, 1995, Noteborn et al., 1991, Noteborn et al., 1994), which can induce apoptosis in human malignant and transformed cell lines, but not in untransformed human cell lines. In vitro, apoptin fails to induce programmed cell death in normal lymphoid, dermal, epidermal, endothelial and smooth-muscle cells. However, when normal cells are transformed they become susceptible to apoptosis by apoptin. (Danen-van Ooschot, 1997 and Noteborn, 1996). Long-term expression of apoptin in normal human fibroblasts revealed that apoptin has no toxic or transforming activity in these cells.

In normal cells, apoptin was found predominantly in the cytoplasm, whereas in transformed or malignant cells i.e. characterized by hyperplasia, metaplasia or dysplasia, it was located in the nucleus, suggesting that the localization of apoptin is related to its activity (Danen-van Oorschot et al. 1997).

Apoptin-induced apoptosis occurs in the absence of functional p53 (Zhuang et al., 1995a), and cannot be blocked by Bcl-2, Bcr-abl (Zhuang et al., 1995), the Bcl-2-associating protein BAG-1 and not by the caspase-inhibitor cowpox protein CrmA (Danen-Van Oorschot, 1997a, Noteborn, 1996).

Therefore, apoptin is a potent agent for the destruction of tumor cells, or other hyperplasia, metaplasia or dysplasia which have become resistant to (chemo)therapeutic induction of apoptosis, due to the lack of functional p53 and (over)-expression of Bcl-2 and other apoptosis-inhibiting agents. (Noteborn et al., 1997).

The fact that apoptin does not induce apoptosis in normal human cells, at least not in vitro, suggests that a toxic effect of apoptin treatment in vivo will be very low. Noteborn et al. (1997) have provided evidence that adenovirus expressed apoptin does not have an acute toxic effect in vivo. In addition, in nude mice it was shown that apoptin has a strong anti-tumor activity.

It appears, that even pre-malignant, minimally transformed cells, may be sensitive to the death-inducing effect of apoptin. In addition, Noteborn and Zhang (1997) have shown that apoptin-induced apoptosis can be used as diagnosis of cancer-prone cells and treatment of cancer-prone cells.

Knowing that apoptin is quite safe in normal cells, but that, as soon as, a cell becomes transformed and/or immortalized (the terms may be used interchangeable herein) the present inventors designed novel means and methods for the induction of apoptosis based on the identification of compounds involved in the apoptin-induced apoptotic cascade.

These compounds are factors of an apoptosis pathway, which is specific for transformed cells. Therefore, these proteins are very important compounds in new treatments and diagnosis for diseases related with aberrancies in the apoptotic process, such as cancer, and auto-immune diseases.

A group of proteins found to be associated with the apoptotic pathway is the family of Bip-like proteins.

Thus the invention provides a recombinant and/or isolated nucleic acid molecule encoding at least a functional part of a member of the family of Bip/GRP78-like proteins comprising at least a functional and/or specific part of the sequence given in figure 1, 2, 3, 4 or 5 or a sequence at least 70%, preferably 80, most preferably 90% homologous therewith. In one possible mechanism of action Bip-like proteins which are chaperones bind to apoptin or apoptin-like proteins resulting in a conformational change in the apoptin-like proteins resulting in enhanced apoptotic activity. Protein -like activity herein is defined as any molecule indirectly or directly providing similar activity as the original protein (in kind not necessarily in amount). It is preferred to bring the Bip-like activity into a cell, which can be done suitably using an expression vector. it is of course preferred if not required that such a cell is also provided with apoptotic, preferably apoptin-like activity. A very suitable manner is to provide apoptin-like activity on another or the same vector.

The invention also provides a recombinant and/or isolated proteinaceous substance having Bip/GRP78-like activty and comprising at least a functional part of the sequence of figure 6 or figure 7 or a functional equivalent thereof or being encoded by a nucleic acid molecule according to claim 1. Except as being used for enhancing apoptosis this proteinaceous substance can also be used to identify further apoptotic agents. Such agents are therefor also part of the present invention.

The invention further provides a method for inducing apoptosis in a cell comprising providing said cell with

Bip/GRP78 inhibiting activity, preferably together with apoptin-like activity.

As stated the invention provides a method for inducing apoptosis through interference with the function of Bip-like proteins (interchangeably referred to as Bip or Bip-like proteins).

The invention provides an anti-tumor therapy based on the interference with the function of Bip-like proteins. The fact that Bip-like proteins are abundantly present in tumor cells in combination with highly expressed oncogenes and that Bip associates to apoptin, make Bip-like proteins very important targets of an anti-tumor agent.

The invention provides Bip as the mediator of apoptin-induced apoptosis, which is tumor-specific.

The invention will be explained in more detail in the following experimental part. This only serves for the purpose of illustration and should not be interpreted as a limitation of the scope of the invention.

EXPERIMENTAL PART

The inventors have used the yeast-2 hybrid system (Durfee et al., 1993) to identify apoptin-associating cellular compounds, that are essential in the induction of apoptosis. The used system is an in-vivo strategy to identify human proteins capable of physically association with apoptin. It has been used to screen cDNA libraries for clones encoding proteins capable of binding to a protein of interest (Fields and Song, 1989, Yang et al., 1992).

Construction of pGBT9-VP3

For the construction of the bait plasmid, which enables the identification of apoptin-associating proteins by means of a yeast-two-hybrid system, plasmid pET-16b-VP3 (Noteborn, unpublished results) was treated with NdeI and BamHI. The 0.4 kb NdeI-BamHI DNA fragment was isolated from low-melting-point agarose.

Plasmid pGBT9 (Clontech Laboratories, Inc, Palo Alto, USA) was treated with the restriction enzymes EcoRI and BamHI. The about 5.4 kb DNA fragment was isolated and ligated with an EcoRI-NdeI linker and the 0.4-kb NdeI-BamHI DNA fragment containing the apoptin-encoding sequences starting from its own ATG-initiation codon. The final construct containing a fusion gene of the GAL4-binding domain sequence and apoptin under the regulation of the yeast promoter ADH was called pGBT-VP3 and was proven to be correct by restriction-enzyme analysis and DNA-sequencing according to the Sanger method (1977).

All cloning steps were essentially carried out as described by Maniatis et al. (1992). The plasmid pGBT-VP3 was prurified by centrifugation in a CsCl gradient and column chromatography in Sephacryl S500 (Pharmacia).

GAL4-activation domain-tagged cDNA libraries

The expression vector pACT, containing the cDNAs from Epstein-Barr-virus-transformed human B cells fused to sequences for the GAL4 transcriptional activation domain,

was used for detecting apoptin-associating proteins. The pACT c-DNA library is derived from the lambda-ACT cDNA library, as described by Durfee et al. 1993.

Bacterial and Yeast strains

The E.coli strain JM109 was the transformation recipient for the plasmid pGBT9 and pGBT-VP3. The bacterial strain Electromax/DH10B was used for the transformation needed for the recovery the apoptin-associating pACT-cDNAs, and was obtained from GIBCO-BRL, USA).

The yeast strain Y190 was used for screening the cDNA library, and all other transformations which are part of the used yeast-two-hybrid system.

Media

For drug selections Luria Broth (LB) plates for E.coli were supplemented with ampicillin (50 microgram per ml). Yeast YPD and SC media were prepared as described by Rose et al. (1990).

Transformation of competent yeast strain Y190 with plasmids pGBT-VP3 and pACT-cDNA and screening for beta-galactosidase activity.

The yeast strain Y190 was made competent and transformed according to the methods described by Klebe et al. (1983). The yeast cells were first transformed with pGBT-VP3 and subsequently transformed with pACT-cDNA, and these transformed yeast cells were grown on histidine-minus plates, also lacking leucine and tryptophan.

Hybond-N filters were layed on yeast colonies , which were histidine-positive and allowed to wet completely. The filters were lifted and submerged in liquid nitrogen to permeabilize the yeast cells. The filters were thawed and layed with the colony side up on Whattman 3MM paper in a petridish with Z-buffer (Per liter: 16.1 gr $Na_2HPO_4.7H_2O$, 5.5 gr $NaH_2PO_4.H_2O$, 0.75 gr KCl and 0,246 gr $MgSO_4.7H_2O$, pH 7.0) containing 0.27% beta-mercapto-ethanol and 1mg/ml X-gal. The

filters were incubated for at least 15 minutes or during night.

Recovery of plasmids from yeast

Total DNA from yeast cells, which were histidine- and beta-galactosidase-positive was prepared by using the glusulase-alkaline lysis method as described by Hoffman and Winston (1987) and used to transform Electromax/DH10B bacteria via electroporation using a Bio-Rad GenePulser according the manufacturer's specifications.

Transformants were plated on LB media containing ampicillin.

Isolation of apoptin-associating pACT clones

By means of colony-filter assay the colonies were lysed and hybridized to a radioactive-labeled 17-mer oligomer, which is specific for pACT (see also section Sequence analysis).

DNA was isolated from the pACT-positive clones, and by means of XhoI digestion analysed for the presence of a cDNA insert.

Sequence analysis

The subclones containing the sequences encoding apoptin-associating proteins were sequenced using dideoxy NTPs according to the Sanger method, which was performed by EuroGentec Nederland BV (Maastricht, The Netherlands). The used sequencing primer was a pACT-specific 17-mer comprising of the DNA-sequence 5'-TACCACTACAATGGATG-3'.

The sequences of the apoptin-associating proteins were compared with known gene sequences from the EMBL/Genbank.

Results and discussion

Apoptin induces specifically apoptosis in transformed cells, such as cell lines derived from human tumors. To identify the essential compounds in this cell-transformation-

specific and/or tumor-specific apoptosis pathway, a yeast genetic screen was carried out.

We have used a human cDNA library, which is based on the plasmid vector pACT containing the complete cDNA copies made from Epstein-Barr virus-transformed human B cells (Durfee et al., 1993).

Construction of a bait plasmid expressing a fusion-gene containing a GAL4 DNA-binding domain and apoptin

To examine the existence of apoptin-associating proteins in the human transformed/tumorigenic cDNA library, a so-called bait plasmid had to be constructed.

To that end, the complete apoptin-encoding region, flanked by about 40 basepairs downstream from the apoptin gene, was cloned in the multiple cloning site of plasmid pGBT9.

The final construct, called pGBT-VP3, was analysed by restriction-enzyme analysis and sequencing of the fusion area between apoptin and the GAL4-DNA-binding domain.

A gene(fragment) encoding an apoptin-associating protein is determined by transactivation of a GAL4-responsive promoter in yeast.

The apoptin gene is fused to the GAL4-DNA-binding domain of plasmid pGBT-VP3, whereas all cDNAs derived from the transformed human B cells are fused to the GAL4-activation domain of plasmid pACT. If one of the cDNAs will bind to apoptin, the GAL4-DNA-binding domain will in the vicinity of the GAL4-activation domain resulting in the activation of the GAL4-responsive promoter, which regulates the reporter genes HIS3 and LacZ.

The yeast clones containing plasmid expressing apoptin and a plasmid expressing an apoptin-associating protein (fragment) can grow on a histidine-minus medium and will stain blue in a beta-galactosidase assay. Subsequently, the plasmid with the cDNA insert encoding the apoptin-associating protein can be isolated and characterized.

Before we could do so, however, we have determined that transformation of yeast cells with pGBT-VP3 plasmid alone or in combination with an empty pACT vector, did not result in the activation of the GAL4-responsive promoter.

Identification of apoptin-associating proteins encoded by cDNAs derived from a human transformed B cell line.

We have found yeast colonies, which upon transformation with pGBT-VP3 and pACT-cDNA were able to grow on a histidine-minus medium (also lacking leucine and tryptophan) and stained blue in a beta-galactosidase assay. These results indicate that these yeast colonies contain besides the bait plasmid pGBT-VP3 a pACT plasmid encoding for a potential apoptin-associating protein.

Plasmid DNA was isolated from these positive yeast colonies, which were transformed in bacteria. By means of an filter-hybridization assay using a pACT-specific labeled DNA-probe, the clones containing pACT plasmid could be determined. Subsequently, pACT DNA was isolated and digested with restriction enzyme XhoI, which is indicative for the presence of a cDNA insert. The pACT plasmids with a cDNA were sequenced.

Description of an apoptin-associating protein

The yeast genetic screen for apoptin-associating proteins resulted in the detection of Bip-like proteins (Bip is also called GRP78 protein).

The determined DNA sequences of the five independent Bip/GRP78 cDNA clones are shown in Figures 1-5, respectively. The combined amino acid sequence of all clones is given in Figure 6, illustrating that they share a common region, which will be the part associating with apoptin.

Construction of an expression vector for the identification of the association of Apoptin and Bip/GRP78-like proteins in transformed mammalian cells.

To study the association of Apoptin and Bip/GRP78-like proteins in a mammalian cellular background, we have generated pSM2NT vectors containing the Bip/GRP78 cDNA inserts. Another important feature of this approach is that we can prove that the cloned cDNA indeed encode an(Apoptin-associating) protein product.

The DNA plasmid pSM2NT contains the adenovirus 5 major late promoter (MLP) and the SV40 ori enabling high levels of expression of foreign genes in transformed mammalian cells, such as Cos cells.

Furthermore, the pSM2NT vector contains a Myc-tag (amino acids: EQKLISEEDL) which is in frame with the foreign-gene product. This Myc-tag enables the recognition of the e.g. Apoptin-associating proteins by means of the Myc-tag-specific 9E10 antibody.

The pSM2NT construct expressing Myc-tagged Bip/GRP78 was constructed as follows. The pACT-Bip/GRP78 clone no. 31 was digested with the restriction enzyme XhoI and the requested cDNA insert was isolated. The expression vector pSM2NT was digested with XhoI and treated with calf intestine alkline phosphatase and ligated to the subsequent isolated cDNA inserts. By sequence analysis, the pSM2NT clone containing the Bip/GRP78 cDNA in the correct orientation were identified.

The expression of the Myc-tagged Bip/GRP78 protein was analyzed by transfection of Cos cells with plasmid pSM2NT-Bip/GRP78. As negative control, Cos cells were mock-transfected.

Two days after transfection, the cells were lysed and Western-blot analysis was carried out using the Myc-tag-specific antibody 9E10. The Cos cells transfected with pSM2NT-Bip/GRP78 were proven to synthesize a specific Myc-tagged Bip/GRP78 product with the expected size of approximately 27 kDa. As expected, the lysates of the mock-

transfected Cos cells did not contain a protein product reacting with the Myc-tag-specific antibodies.

These results indicate that we have been able to isolate a cDNA that indeed is able to produce a Bip/GRP78-like protein product with the ability to associate to the apoptosis-inducing protein Apoptin.

Co-immunoprecipitation of Myc-tagged Bip/GRP78 with Apoptin in a transformed mammalian cell system.

Next, we have analyzed the association of Apoptin and Bip/GRP78 by means of co-immunoprecipitations using the Myc-tag-specific antibody 9E10. The 9E10 antibodies were shown not to bind directly to Apoptin, which enables the use of 9E10 for carrying out co-immuno-precipitations with (myc-tagged) Apoptin-associating proteins and Apoptin.
To that end, Cos cells were co-transfected with plasmid pCMV-VP3 encoding Apoptin and with plasmid pSM2NT-Bip/GRP78 encoding the Myc-tagged Bip/GRP78 protein. As negative control, we have transfected cells with Apoptin and a plasmid pSM2NT-LacZ encoding the myc-tagged beta-galactosidase, which does not associate with Apoptin.

Two days after transfection, the cells were lysed in a buffer consisting of 50 mM Tris (7.5), 250 mM NaCl, 5 mM EDTA, 0.1 % Triton X100, 1 mg/ml Na4P2O7 and freshly added protease inhibitors such as PMSF, Trypsine-inhibitor, Leupeptine and Na3VO4. The specific proteins were immuno-precipitated as described by Noteborn et al. (1998) using the Myc-tag-specific antibodies 9E10, and analyzed by Western blotting.

Staining of the Western blot with 9E10 antibodies and 111.3 antibodies, which are specifically directed against Apoptin, showed that the `total' cell lysates contained Apoptin and the Myc-tagged Bip/GRP78 or beta-galactosidase product. Immunoprecipitation of the Myc-tagged Bip/GRP78 products was accompanied by the immunoprecipatation of Apoptin product of 16 kDa. In contrast, immunoprecipitation

of myc-tagged beta-galactosidase did not result in co-precipitation of the Apoptin protein.

In total, three independent immunoprecipitation experiments were carried out, which all showed the associating ability of Apoptin to the Bip/GRP78 proteins.

These results indicate that besides the yeast background, Bip/GRP78 is able to specifically associate with Apoptin in a mammalian transformed cellular system.

Characteristics of Bip/GRP78-like proteins

Glucose regulated proteins are, like heat shock proteins, induced by stress. The most abundant glucose regulated protein is GRP78 (78 kD), also known as the immunoglobulin heavy chain binding protein Bip. It functions as a molecular chaperone and binds Ca^{2+}. It is expressed in many cell types and is located in the endoplasmic reticulum (ER; Lee, 1992).

Bip is permanently highly expressed in progressively growing tumors. Furthermore, Bip levels were increased in murine embryonic cells transformed by chemicals or radiation, and the level of Bip in fibrosarcomas was found to correlate with tumor growth (Gazit et al., 1995).

In Chinese hamster ovary cells, inhibition of Bip leads to increased cell death during chronic hypoxia or after treatment with a Ca2+ ionophore. In fibrosarcoma cells, Bip protects against cell lysis induced by cytotoxic T lymphocytes (CTLs) and tumor necrosis factor (TNF), suggesting that increased levels of Bip may protect tumor cells from immune attack <u>in vivo</u> (Sugawara et al., 1990).

In B/C10ME fibrosarcoma cells, inhibition of Bip by an anti-sense construct results in increased apoptotic cell death after Ca2+ depletion from the ER, but the <u>in vitro</u> growth rate is not affected. Upon injection of these cells in mice, no tumors were formed (Jamora et al., 1996).

Jamora et al. (1996) have suggested that suppression of Bip may be a new approach to cancer therapy. Their data, however, do not prove the application of such a therapy.

The fact that Bip associates with apoptin, makes apoptin and/or Bip essential elements of a feasible anti-tumor therapy (Noteborn et al., 1997).

Therefore, the interference of Bip by, e.g. apoptin, in transformed cells is the crucial event during anti-tumor therapy. We have found an example of an effective regulator of Bip-like proteins, resulting in induction of apoptosis, which is the key to tumor rejection.

Induction of apoptosis through interference of Bip-like proteins

Our results indicate that apoptin can change and/or eliminate the Bip-like mediated activity, resulting in induction of apoptosis. This mechanism is one possibility of action (Jamora et al., 1996).

Bip-like proteins are chaperone proteins, which can influence the conformation of proteins and by doing so its function. Association of apoptin with Bip-like proteins will result in a change of its conformation and its function. Apoptin will be able to enter and/or to stay in the nucleus of (transformed) cells, and as a consequence the cell will undergo apoptosis.

It is known that apoptin induces apoptosis preferentially in transformed cells and/or cells expressing transforming agents, most likely due to interaction with specific Bip-like proteins.

Co-expression of Bip-like protein and apoptin in normal cells results in induction of apoptosis

Next, we have examined the effect of expression of Bip-like proteins and apoptin on the induction of apoptosis in normal cells. To that end, VH10 and VH25 cells were transfected with plasmids encoding Bip-like protein and apoptin (Graham and Van der Eb, 1973). By immunofluorescence apoptin- and/or Bip specific monoclonal antibodies (Noteborn et al., Van den Heuvel, 1990) apoptin- and Bip-positive cells were detected.

The percentage of apoptotic cells within the group of apoptin and/or Bip-like protein-positive cells was detected staining the cells with DAPI (Danen-Van Oorschot et al., 1997, Telford et al., 1992). The normal fibroblasts expressing apoptin or Bip alone did not undergo apoptosis, whereas the cells co-expressing both apoptin and Bip did.

Factors that make use of interference with the function of Bip-like proteins, which results in induction of apoptosis are apoptin and apoptin-like proteins. Furthermore, it concerns proteins that are related to apoptin-induced apoptosis, such as the CAV-derived protein VP2, which is known to enhance apoptin-induced apoptosis (Noteborn et al., 1997).

Other apoptin-associating proteins

The genetic yeast screen with pGBT-VP3 as bait plasmid and pACT plasmid containing cDNAs from transformed human B cells also delivered the protein filamin. The protein filamin is localized within lamellipodia and filopodia. Filamin is one of the cross-linking proteins of actin. It may play an additional role of linking the cytoskeleton to cell-substratum adhesion sites (Matsudaira, 1994).

Two independent filamin-like clones were found. The found associating amino acid sequence of the two filamin clones are shown in Figure 7.

To analyze into further detail the associating properties of Apoptin and filamin, we have co-expressed Myc-tagged filamin-like proteins by means of the pSM2NT vector (as described for Bip/GRP78) in Cos cells together with Apoptin.

Immunoprecipitation data clearly showed that 9E10 precipitates both filamin and Apoptin indicating that Apoptin associates to filamin in Cos cells.
Our data indicate that Apoptin associates with filamin in both yeast and transformed mammalian cells.

Another apoptin-associating protein that was found is a TPR-1-like protein. In total four independent pACT-cDNA

clones could be determined. TPR-1 (Murthy et al., 1996) was indentified by its ability to bind to neuro-fibromin. It contains three tandem tetratricotpeptide motifs (Blatch et al., 1997), but shows no homology outside this domain to other known proteins. The combined amino acid sequence of the observed TPR-1 clones is shown in Figure 8.

Also, a human homolog of the bacterial chaperone DNAJ (Schlenstedt et al., 1995) was found as an apoptin-associating protein. The DNA sequence of the observed DNAJ-like clone is shown in Figure 9.

To analyze into further detail the associating properties of Apoptin and this DNAJ-like protein, we initially have expressed Myc-tagged DNAJ-like cDNA (clone 26; see figure 9) by means of the pSM2NT vector (as described for Bip/GRP78) in Cos cells. Western-blot analysis using the Myc-tag-specific antibodies 9E10 showed a specific Myc-tagged DNAJ-like protein of 30 kDa. These results indicate that the isolated cDNA indeed encodes a protein of the expected size.

Next immunoprecipitation assays were carried out with transiently transfected Cos cells co-synthesizing Myc-tagged DNAJ and Apoptin. The results clearly showed that 9E10 precipitates both DNAJ-like proteins and Apoptin indicating that Apoptin associates with this new DNAJ-like protein in a mammalian transformed background. In total, three independent immunoprecipitation experiments were carried out, which all showed the associating ability of Apoptin to the DNAJ-like proteins.

In summary, our findings prove that our newly discovered DNAJ-like protein is able to associate to the apoptosis-inducing protein Apoptin in both a yeast and mammalian cellular background. Therefore, this DNAJ-like protein plays an important role in the induction of (Apoptin-regulated) apoptosis.

Other DNAJ-like domains (indirectly) also activate Apoptin-induced apoptosis in non-transformed human cells.

Co-expression of SV40 large T antigen (LT) an Apoptin results in apoptin-induced apoptosis in normal diploid cells derived from human individuals and rodents (Noteborn and Zhang, 1998). These data prove that diploid cells are not susceptible to Apoptin, whereas they become when they express a transformning protein.

In a new series of experiments, we have investigated the effect of mutations within SV40 LT on the level of Apoptin-induced apoptosis in normal diploid human fibroblasts.

To that end, human VH10 fibroblasts were co-transfected with plasmids encoding Apoptin and complete LT, LT-mutant 3213, lacking the Retinoblastoma-binding site, LT-mutant 5031 lacking p53-binding sites, LT-mutant 1135 minus the J-domain or the LT-mutant 136 containing almost only the J-domain sequences and the nuclear location signal of SV40 (Srinivasan, 1997). This SV40 DNAJ-like domain harbors transforming activity and is involved in DNA replication, which is similar to the E. coli DNAJ activity is involved in lambda bacteriophage DNA replication (Campbell et al., 1997).

The LT mutants lacking the Retinoblastoma and p53-binding sites were shown to translocate Apoptin into the nucleus and activate the Apoptin-induced apoptosis to the same extent as the wild-type LT (Noteborn and Zhang, 1998; Figures 10 and 11). These data indicate that both p53 and Retionoblastoma gene products are not relevant for Apoptin-induced apoptosis, which confirms our previous data (Zhang et al., 1995).

In contrast to the LT mutant with the DNAJ-domain deletion, which causes significantly less Apoptin activity in the normal VH10 fibroblasts. This feature shows the importance of the LT DNAJ-like domain on the Apoptin activity. These results are strenghtened by the fact that the LT mutant containing almost only the DNAJ-like domain activates Apoptin completely and translocates it optimally into the nucleus (Figures 10 and 11).

The SV40 DNAJ-like domain is not homologous to the Apoptin-associating sequences of our newly cloned DNAJ-like cDNA. This observation is in agreement with the fact that Apoptin does not directly associate with the SV40 LT DNAJ-like domain, as detected in co-immunoprecipitations.

Therefore, we conclude that DNAJ-like proteins have at least two independent domains playing a role in Apoptin-induced apoptosis. Nevertheless, these results show the obvious relationship of Apoptin and DNAJ-like activity in the ability of Apoptin induction of apoptosis.

Differential post-translational modification patterns of Apoptin in transformed/tumorigenic cells versus normal cells.

The fact that Apoptin is active in a transformed/tumorigenic cellular background, due to DNAJ-like activities and/or other agents, let us conclude to determine a possible differential modificational characteristic of Apoptin in human transformed/tumorigenic cells versus normal cells.

Therefore, we have carried out a kinase-reaction assay on a bacterial-produced Apoptin protein and lysates obtained from transformed/tumorigenic human cells (Saos-2 cells; Zhuang et al., 1995) or from normal non-transformed VH10 cells. Apoptin incubated with lysate derived from the transformed/tumorigenic human cells was labeled with 32P, whereas Apoptin with lysates derived from normal non-transformed cells was not. These results indicate that (human) transformed/tumorigenic cells harbor an Apoptin-specific kinase activity, which is absent in non-transformed (human) cells.

The fact that Apoptin is differentially post-translationally modified in transformed/tumorigenic versus normal non-transformed cells, forms the base of a diagnostic assay for the determination of transformed/tumorigenic material derived from patients with suspicious tissue.

The advantage of such a method is that one does not need to culture primary (tumor) cells under tissue-culture conditions. Most of the cases, isolated primary (tumor) cells will hardly grow under these conditions.

Production of polyclonal antibodies directed against DNAJ like proteins.

For the production of polyclonal antibodies against DNAJ-like proteins a putative immunogenic peptide was synthesized (N-terminus-RNKPVARQAPGKRKC-C/terminus; EuroGentec SA, Belgium). Subsequently, rabbits were injected with the specific peptides according the standard procedures of the manufacturer.

The serum derived from the rabbits injected with the DNAJ-like peptide was shown to be specific for in this report described DNAJ-like products by means of immuno-fluoresence and Western-blot assays.

These results imply that we have generated specific antibodies, which can be used for detecting our discovered DNAJ-like Apoptin-associating protein.

Description of the figures

Figure 1 shows the DNA sequence of the analysed region of the apoptin-associating clone Bip/GRP78 No-1.

Figure 2 shows the DNA sequence of the analysed region of the apoptin-associating clone Bip/GRP78 No-2.

Figure 3 shows the DNA sequence of the analysed region of the apoptin-associating clone Bip/GRP78 No-3.

Figure 4 shows the DNA sequence of the analysed region of the apoptin-associating clone Bip/GRP78 No-4.

Figure 5 shows the DNA sequence of the analysed region of the apoptin-associating clone Bip/GRP78 No-5.

Figure 6 shows the combination of the amino acids of the sequenced Bip-like clones No-1 till No-5. The fact that they overlap with each other implies that the common region of all five inserts will associate with apoptin. The amino acid sequence of the known Bip/GRP78 is also shown.
In addition, the three C-terminal amino acids H-E-G of the multiple cloning site of pACT are given to illustrate that the Bip/GRP78-like amino acid sequence is in frame with the GAL4-activation domain. This feature proves that the Bip/GRP78-like region is indeed synthesized.

Figure 7 shows the amino acids of the sequenced region of the apoptin associating clone Filamin No-1 and No-2. In addition, the three C-terminal amino acids H-E-G of the multiple cloning site of pACT are given to illustrate that the filamin-like amino acid sequence is in frame with the GAL4-activation domain. This feature proves that the filamin-like region is indeed synthesized.

Figure 8 shows the amino acid sequence, derived from the analysed region of the apoptin-associating clones TRP-1 No1 till No4. Also, the known TRP-1 (EMBL/Genbank) is shown.

Figure 9 shows the DNA sequence of the analysed region of the apoptin-associating clone DNAJ-like protein.

Figure 10 shows the induction of apoptosis due to co-expression in normal non-transformed VH10 cells of Apoptin and Desmin (VP3+des; negative control), wild-type SV40 LT (VP3+LT), LT-mutant lacking the retinoblastoma-binding site (VP3+3213), LT-mutant missing the p53-binding sites (VP3+5031), LT-mutant with a deletion in the J domain (VP3+1135), and LT-mutant 136 containing almostonly the J domain or a nuclear localization signal (VP3+136). Actually, the % of apoptosis observed with desmin and Apoptin resembles the background level due to the transfection procedures (Danen-Van Oorschot, 1997).

Figure 11 shows the nuclear localization of Apoptin due to co-expression, as described also for Figure 10, in normal non-transformed VH10 cells of Apoptin and Desmin (VP3+des; negative control), wild-type SV40 LT (VP3+LT), LT-mutant lacking the retinoblastoma-binding site (VP3+3213), LT-mutant missing the p53-binding sites (VP3+5031), LT-mutant with a deletion in the J domain (VP3+1135), or LT-mutant 136 containing almost only the J domain and a nuclear localization signal (VP3+136). Actually, the % of nuclear localization observed with desmin and Apoptin resembles the background level due to the transfection procedures (Danen-Van Oorschot, 1997).

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cells but not in normal cells as essential characteristic for the development of an anti-tumor therapy.

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CLAIMS

1. A recombinant and/or isolated nucleic acid molecule encoding at least a functional part of a member of the family of Bip/GRP78-like proteins comprising at least a functional and/or specific part of the sequence given in figure 1, 2, 3, 4 or 5 or a sequence at least 70%, preferably 80, most preferably 90% homologous therewith.

2. An expression vector comprising a nucleic acid molecule according to claim 1.

3. An expression vector according to claim 2 further comprising a nucleic acid sequence encoding apoptin-like activity.

4. A recombinant and/or isolated proteinaceous substance having Bip/GRP78-like activty and comprising at least a functional part of the sequence of figure 6 or figure 7 or a functional equivalent thereof or being encoded by a nucleic acid molecule according to claim 1.

5. A method for identifying apoptotic agents comprising the use of a proteinaceous substance according to claim 4.

6. An apoptotic agent obtainable by a method according to claim 5.

7. A method for inducing apoptosis in a cell comprising providing said cell with Bip/GRP78 inhibiting activity.

8. A method for inducing apoptosis in a cell comprising providing said cell with apoptin-like activity together with Bip/GRP78 activity.

9. A method according to claim 8 whereby said activities are provided by nucleic acid molecules encoding said activity.

CAGCTTCTGATAATCAACCAACTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCCTGACAAAA
GACAATCATCTTCTGGGTACATTTGNTCCGACTGGAATTCCTCCTGCTCCTCGTGGGGTCCCACA
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ACAGGGAACANAAATAAGATCACAATCACCAATGACCAGAATCGCCTGACACCTGAAGAAATCNA
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TGGNTGGAAAANCAACCAATATGCNGACTTNNAAANTTCAANGNTAAGANNAGGGAANTGGGAAN
AATTTTTCACCCATTTTNCNAGNANACCCTANGNANTTNNAAGGCCCCCCCCCAATTNGGTANAG
GGGTTCCACCANAANAAATNGTTTTNTCACNCGGTTTCNGANNNGNCTNTTAANNTTGTAAAATN
GGGGCCCCNTT

Figure 1

CAGCTTCTGATAATCAACCAACTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCCTGACAAAA
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TTGTTCANCCAATTATCAGCAAACTCCAATGGAAGTGCAAGCCCTCCCCCAACTGGTGAAGANGA
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Figure 2

CTGATAATCAACCAACTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCCTGANAAAAGACAAT
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NCTNCTCGNNAACTGGGTGANTNAGGTTTCANCAAGANAAANTATTAAGTTTGNTAGNNACNGGA
TCTGGNTANGTGNCTGTANAANTGGTNTANTACGGNGNCTCAANGGAACTTAG

Figure 3

GGCCACGAAGGCCCACAGTGGTGCCTACCAAGAAGTCTCAGATCTTTTCTACAGCTTCTGATAAT
CAACCAACTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCCTGACAAAAGACAATCATCTTCT
GGGTACATTTGATCTGACTGGAATTCCTCCTGCTCCTCGTGGGGTCCCACAGATTGAAGTCACCT
TTGAGATAGATGTGAATGGTATTCTTCGAGTGACAGCTGAAGACAAGGGTACAGGGAACAAAAAT
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CTCTGAAGATAAGGAGACCATGGAAAAAGCTGTAGAAGAAAAGATTGAATGGCTGGAAAGCCACC
ATGATGCTGACATTGAAGACTTCAAAGCTAAGAAGAAGAACTGGAAGAAATTGTTCAACCAATTA
TCAGCAAACTCTATGGGAANTGNAGGCCTCCCT

Figure 4

CTGATAATCAACCAACTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCCTGACAAAAGACAATCATCTTC
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Figure 5

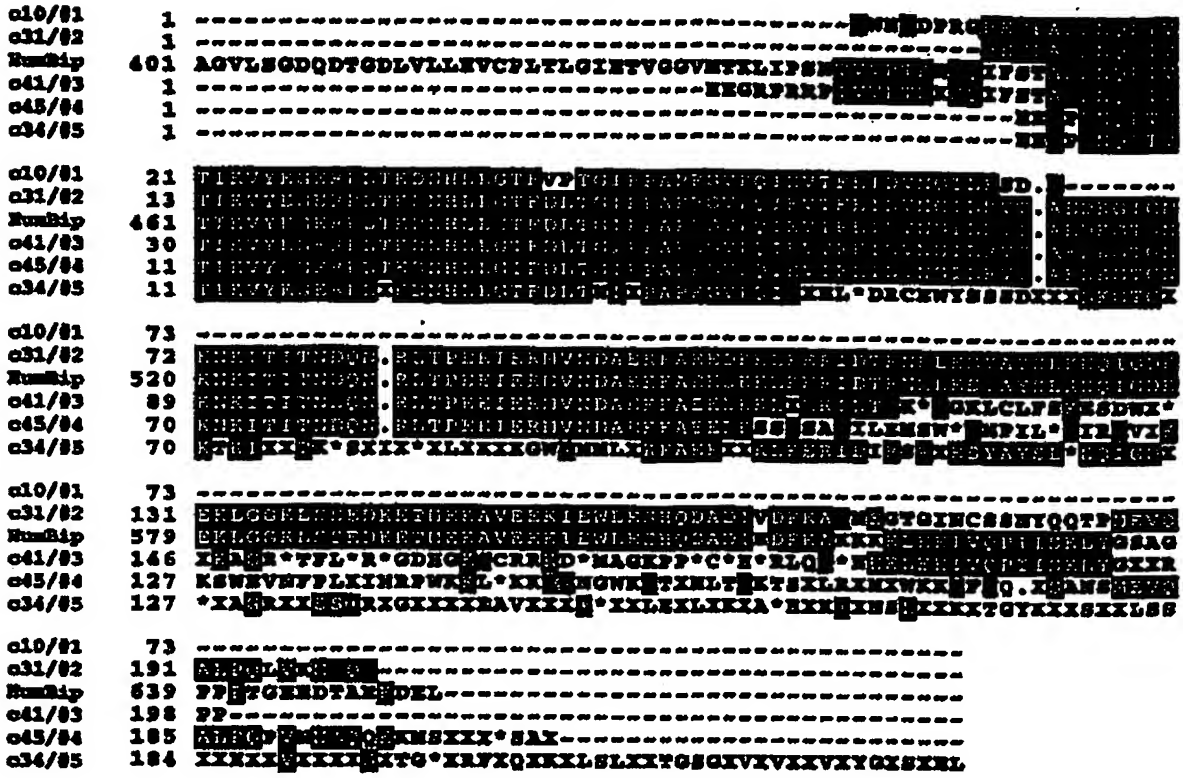
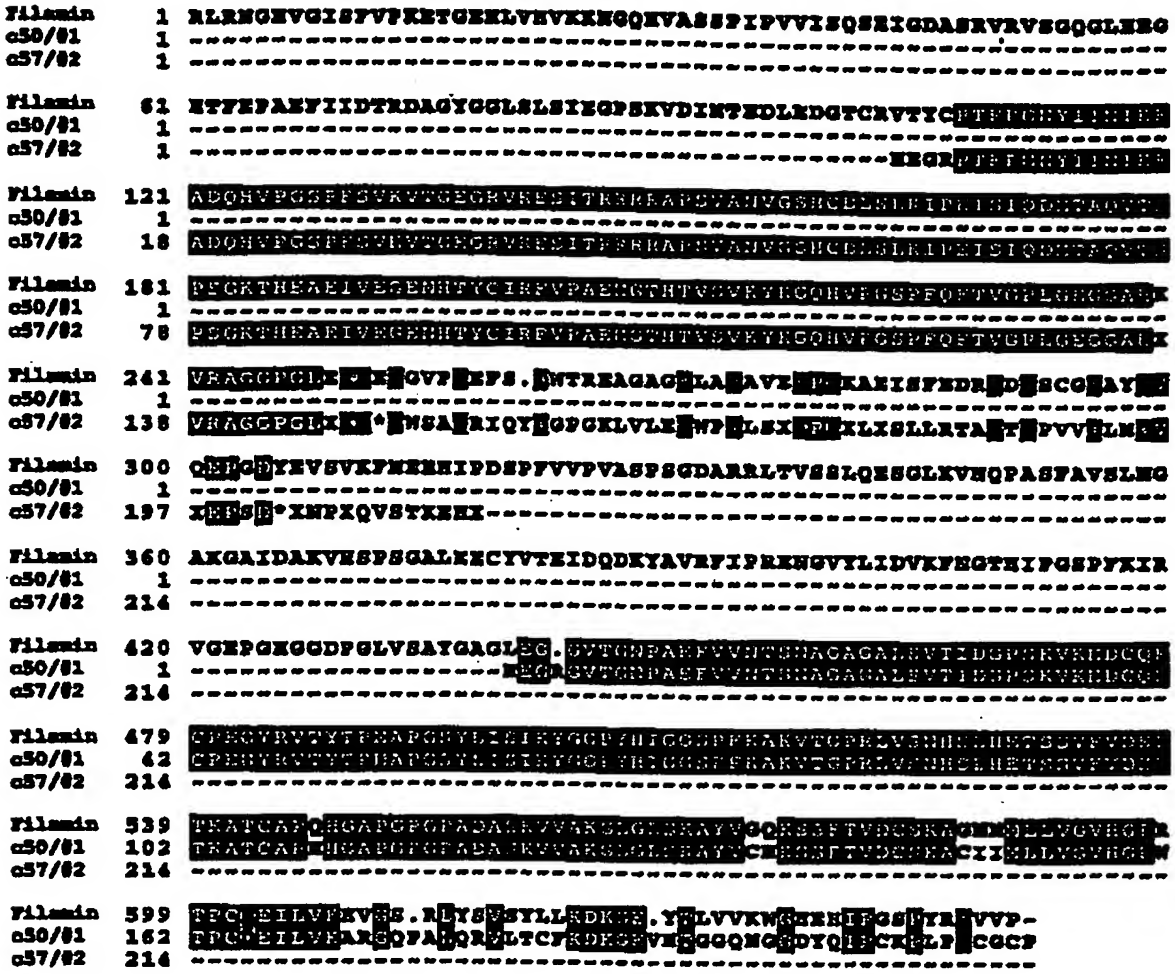


Figure 6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Filamin 360
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Figure 7

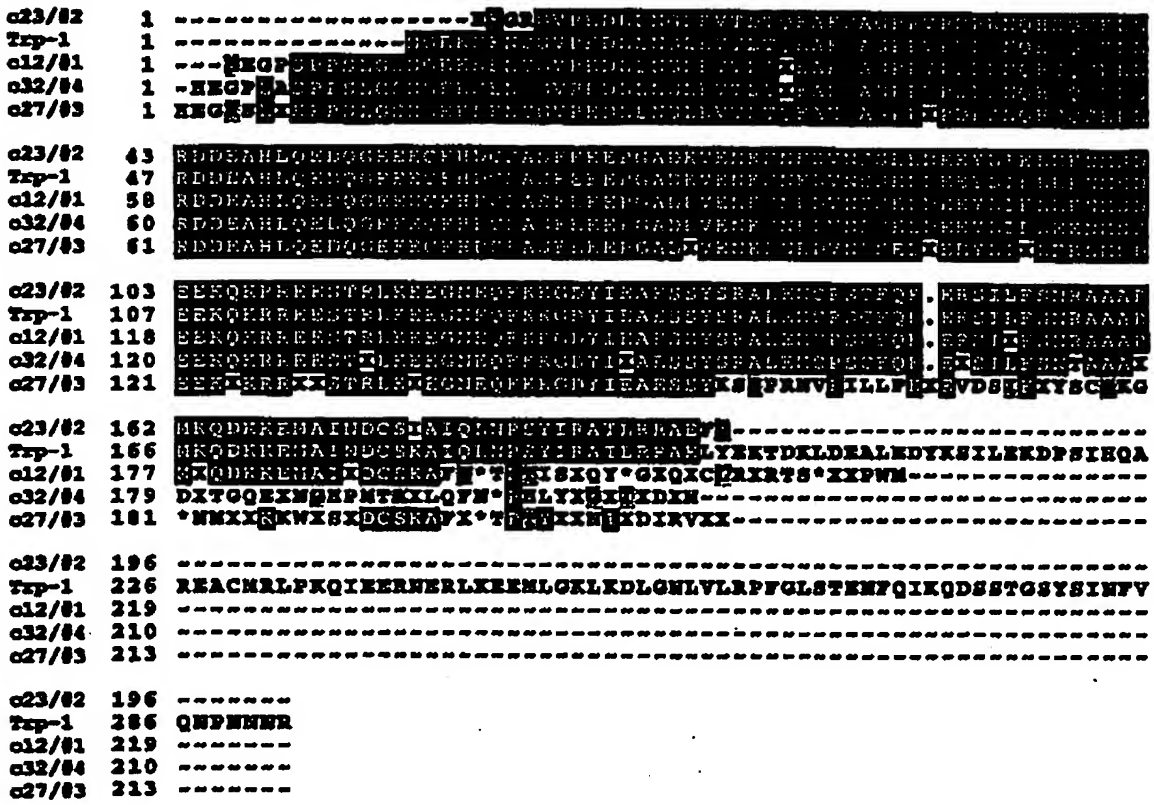

Figure 8

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Figure 9

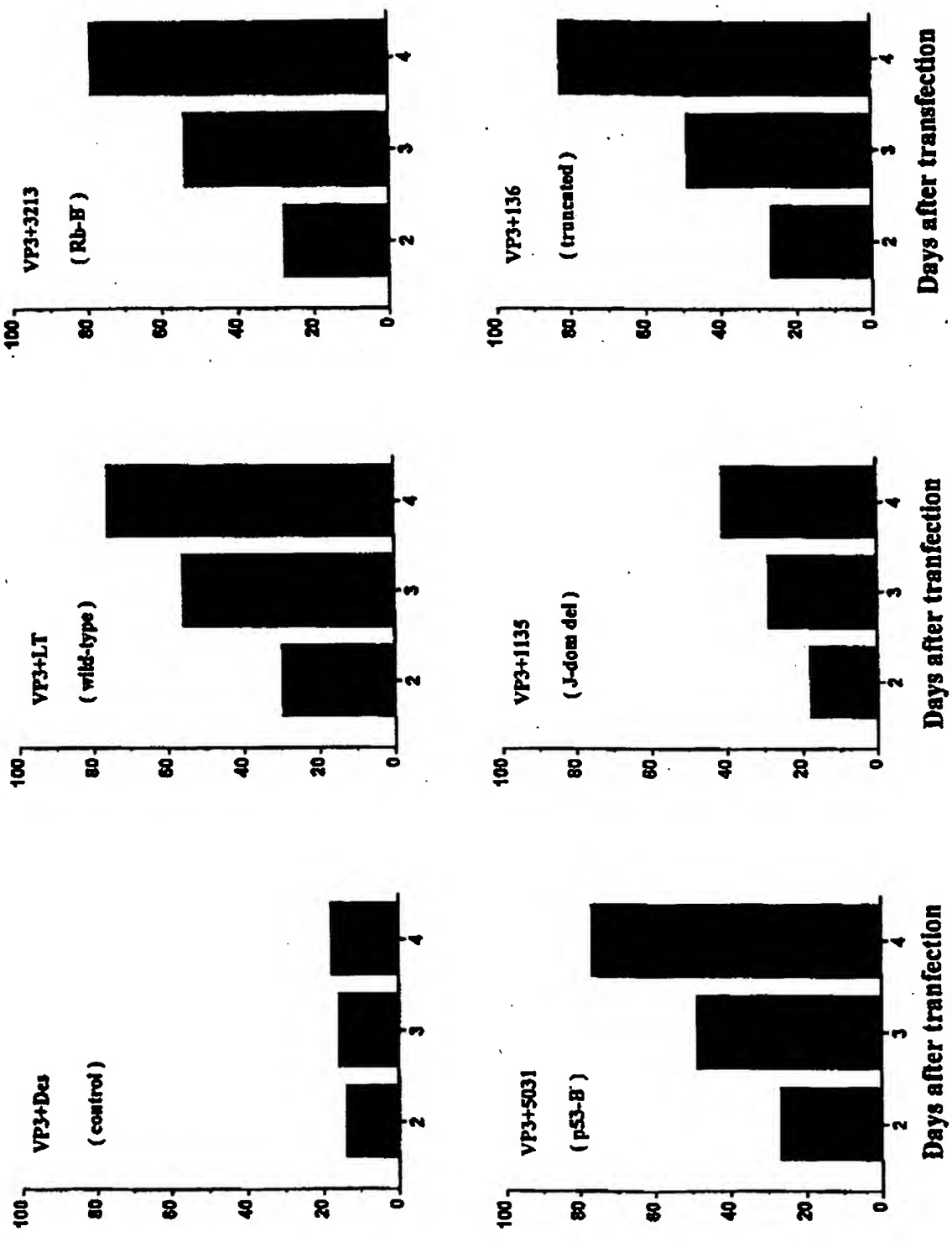
VP3+Dss
(control)
VP3+LT
(wild-type)
VP3+1135
(J-dom del)
VP3+3213
(Rb-B')
VP3+5031
(p53-B')
VP3+136
(truncated)
Apoptin-induced apoptosis (%)
Days after tranfection
Days after transfection

Figure 10

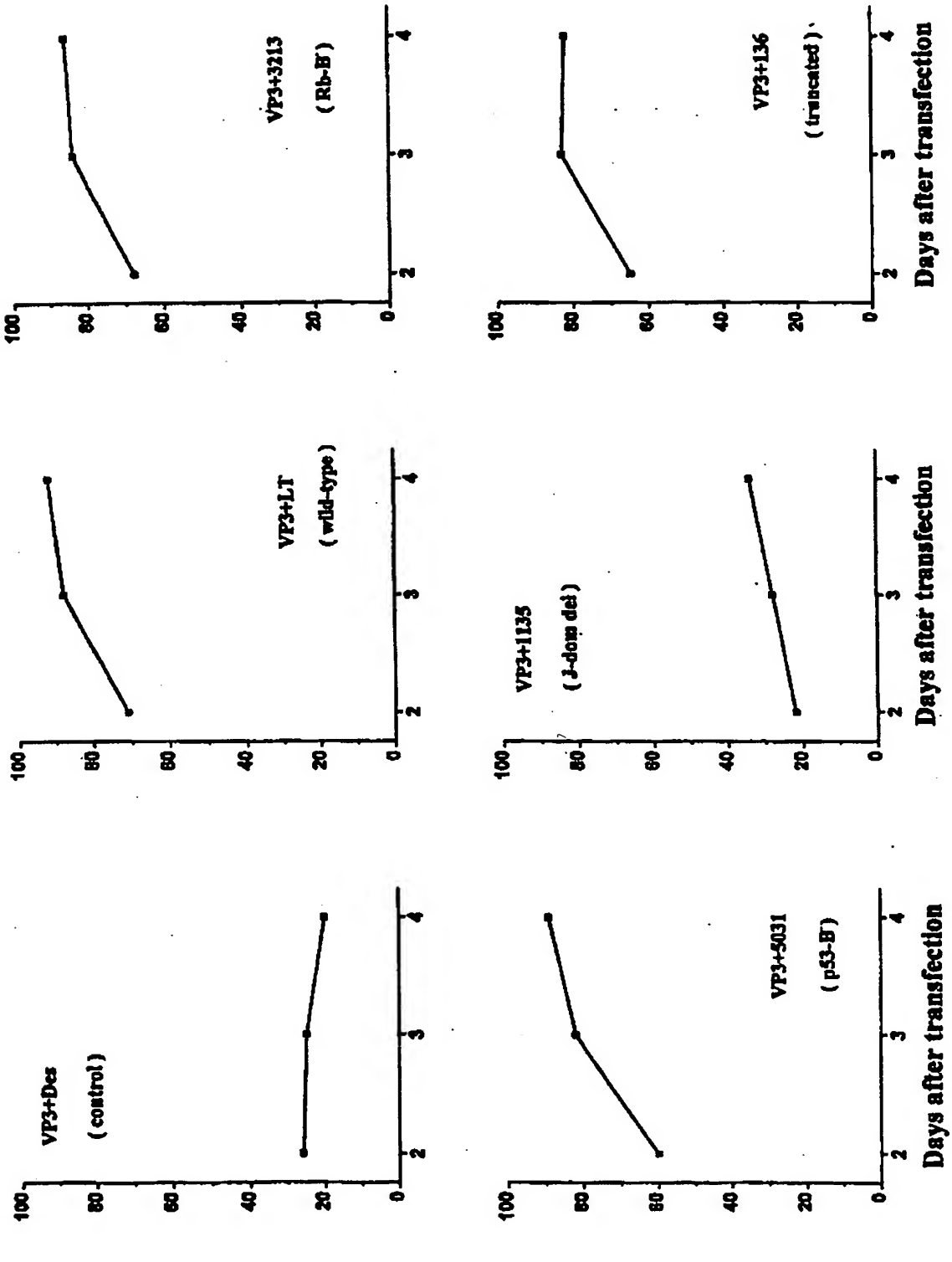
VP3+Dcs
(control)
VP3+LT
(wild-type)
VP3+3213
(Rb-B')
VP3+5031
(p53-B')
VP3+1135
(J-dom del)
VP3+136
(truncated)
Nuclear localization of Apoptin (%)
Days after transfection
Days after transfection

Figure 11